

# The term phytase comprises several different classes of enzymes

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## Dedication

This minireview on phytases' classification is dedicated to honor Professor I.C. Gunsalus, a great mentor to one of us.<sup>1</sup> Several of the research findings from our laboratory had reached his office in the form of manuscripts as editor of *Biochemical Biophysical Research Communications*. Professor Gunsalus supported our work by encouraging us to publish in a timely fashion. He leaves a legacy of outstanding research and a willingness to encourage others to conduct innovative work. We thank him for encouraging us to push the boundary of phytase research over the last decade.

## Introduction

Phytase is a generic term used to describe an enzyme that hydrolyzes phosphomonoester bonds from phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), thereby liberating inorganic orthophosphates [1]. The number of enzymes described as phytase or phytate-degrading enzymes has increased over the last decade [2]. However, not all of these enzymes are structurally similar nor do they all cleave phosphate groups from phytate with the same mechanism. The varied catalytic properties and requirements observed in these enzymes

reflect different methods by which organisms dephosphorylate phytate to produce *myo*-inositol phosphates. Research on the active site geometry and the mechanism is emerging. Consequently, this knowledge allows us to assign these enzymes to specific classes of enzymes.

In order for an enzyme to be a phytase it must display phosphatase activity. Depending on the pH versus activity profile and the optimum pH for catalysis, these enzymes are further broadly classified as acid, neutral, or alkaline phosphatases. Since most of the recent interest generated in phytase research had centered on identifying an enzyme that would function effectively in the digestive tract of monogastric animals, most of these studies have focused on acid phosphatases with high specific activity for the preferred substrate, phytic acid. Within this subdivision, three structurally distinct classes of enzymes have been described to date as phytases. These three classes include representatives of histidine acid phosphatases (HAP),  $\beta$  propeller phytase (BPP), and purple acid phosphatases (PAP) (Table 1). In this minireview, examples and unique properties of each group will be presented.

## Histidine acid phosphatase phytases

Most known phytases are HAPs (EC 3.1.3.8). All members of this class share both a common active site motif, RHGXRXP, and a two-step mechanism that hydrolyzes phosphomonoesters [3,4]. However, not all HAPs are catalytically active as phytases. Recent research [5] has established a vital role for the enzyme's substrate specificity site (SSS). Even among known HAP phytases, specific activity for phytic acid varies a great deal. For example, Wyss and co-workers [6] compared the catalytic properties of several fungal phytases and proposed two classes of HAP phytases. One class has a broad substrate specificity but a lower specific activity for phytic acid and the second class has a narrow sub-

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<sup>1</sup> A.H.J.U. is grateful to Professor Gunsalus for the opportunity to work in his laboratory as a postdoctoral fellow for over three years in the early 1980s on linalool 8-methyl hydroxylation system of *Pseudomonas incognita*. The training that I received during my tenure at Urbana, Illinois, shaped my future research activities on phytase at USDA's Southern Research Research Center at New Orleans. Research on phytase is expanding because of its linkage to animal nutrition, eutrophication, and phosphate depletion from soil. Research ideas obtained in Professor Gunsalus's laboratory when applied to phytase research in the mid-1980s made a significant impact.

Table 1  
Classes of phytases

Enzyme class	Examples	Molecular mass of monomer (kDa)	NCBI structure No.
HAP	PhyA	85 <sup>a</sup>	1IHP
	PhyB	68 <sup>a</sup>	1QFX
	AppA	45 <sup>b</sup>	1DKP
BPP	TS-Phy	44 <sup>b</sup>	1H6L
	PhyC	43 <sup>b</sup>	—
PAP	GmPhy	72 <sup>a</sup>	—

<sup>a</sup> Glycosylated.

<sup>b</sup> Nonglycosylated.

strate specificity but a high specific activity for phytate. No significant variation in the catalytic centers of these fungal phytases has thus far been discovered. However, when the amino acid residues comprising the SSS are examined [5,7], a pattern of low specific activity for phytase and a neutral amino acid occupying the residue analogous to K300 in *Aspergillus niger* NRRL 3135 phytase (phyA) is evident [8]. When a three-dimensional model of the phyA molecule was examined, the six amino acids in its substrate specificity site, K91, K94, E228, D262, K300, and K301, were found to encircle the cavity containing the HAP active center [7]. They thus appear to have a role as a gate-keeper for any substrate's access to the HAP active site. *A. niger* NRRL 3135 phytase (phyA) is widely known for its high specific activity for phytic acid [9] and is commercially marketed as Natuphos as an animal feed additive to lower phosphate levels in manure from poultry and swine. Kostrewa and co-workers [5] explain this high specific activity for phytate by revealing its SSS that is optimized for binding the negatively charged phytic acid. This model also offers a reason that other less charged substrates do not bind as effectively to the SSS of *A. niger* NRRL 3135 phyA. A three-dimensional model of *A. niger* NRRL 3135 phyA (1IHP) from the National Center for Biotechnology Information's (NCBI) Web site is shown in Fig. 1A.

A second phytase (phyB) has been isolated from *A. niger* NRRL 3135 [10]. Its catalytic properties are distinct from those of *A. niger* NRRL 3135 phyA. In fact, it first appeared in the literature not as a phytase but as an *A. niger* acid phosphatase optimum pH 2.5 [11]. When it was first isolated and tested for phytase activity, the activity assay was conducted at pH 5.0, the optimum pH for *A. niger* NRRL 3135 phyA. However, the pH optimum for phyB is lower, pH 2.5, and no or only minimal activity is observed at pH 5.0. This lack of activity at pH 5.0 for phyB can now be understood by examining its SSS. Kostrewa and co-workers [5] identified the SSS of phyB, which is different from the SSS of phyA. It is composed of just two amino acids; in *A. niger* T213 phyB they are D75 and E272. This means the phyB SSS



Fig. 1. Computer-generated molecular models from National Center for Biotechnology Information (NCBI)'s Web site ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) of representatives from three classes of phosphatases: (A) 1IHP, PhyA, a histidine acid phosphatase; (B) 1H6L, Ts-Phy, a  $\beta$  propeller phytase; (C) 1KBP, KSPAP, a purple acid phosphatase.

is more electrostatically neutral and therefore can accommodate a broader substrate spectrum [5,12] than *A. niger* phyA. It also means that at pH 2.5 the SSS of *A. niger* T213 phyB is uncharged and will accept negatively charged phytate as a substrate; however, at pH 5.0, it is negatively charged and would thus repel a negatively charged substrate [5].

Another difference between *A. niger* phyA and phyB is that the active form of phyA is a monomer, whereas the active form of phyB is a tetramer [12]. This tetrameric structure initially provides phyB with thermostability, but it also explains why, unlike phyA, it is incapable of proper refolding after it has been denatured by heating. The individual protomers are apparently unable to properly reassociate into an active form after the phyB tetramer is denatured.

Thus far, phyA (HAP) has been isolated from filamentous fungi, bacteria, yeast, and plants [7]. Because of the proven efficacy of phyA as an animal feed additive several of these phyA (HAP) phytases (Natuphos, RoNozyme, etc.) are now being marketed. These com-



Fig. 1. (continued)

mercial phytases are produced by large-scale fermentation coupled with overexpression. PhyA (HAP) has also been overexpressed in several transgenic plants [13]. Such transgenic plants have been suggested as a potential alternative method of phytase production for the animal feed industry [14] or a means of developing plant cultivars that require less phosphorus fertilizer [15]. Recently, the HAP phytase gene from *E. coli*, *appA*, has also been successfully expressed in a transgenic pig [16].

The phyB (HAP) has only been reported from *Aspergillus niger* [17,12] and *Aspergillus awamori* [18]. No commercial form of this enzyme is currently marketed.

### $\beta$ Propeller phytase

Unlike HAP, BPP (EC 3.1.3.8) is a recently discovered class of enzyme with a novel mechanism for

hydrolyzing its substrate. BPPs have been isolated and their genes cloned from *Bacillus subtilis* (phyC) [19] and *Bacillus amyloliquefaciens* (TS-Phy) [20]. A three-dimensional model of its molecule displays a basic form similar to a propeller with six blades [21]. A search of the Protein Data Base revealed no other known phosphatase with this type of structure. The dependence on binding  $\text{Ca}^{2+}$  for thermostability and catalytic activity further distinguishes phyC from other subclasses of phytases [22]. BPP has two phosphate binding sites [23]. The hydrolysis of its substrate occurs at the “cleavage site” and the adjacent “affinity site” which increases the binding affinity for substrates like phytic acid that feature neighboring phosphate groups. The calcium ions facilitate the binding by creating a favorable electrostatic environment [22]. Fig. 1B is a three-dimensional model of  $\beta$  propeller phytase (1H6L) from the NCBI Web site.

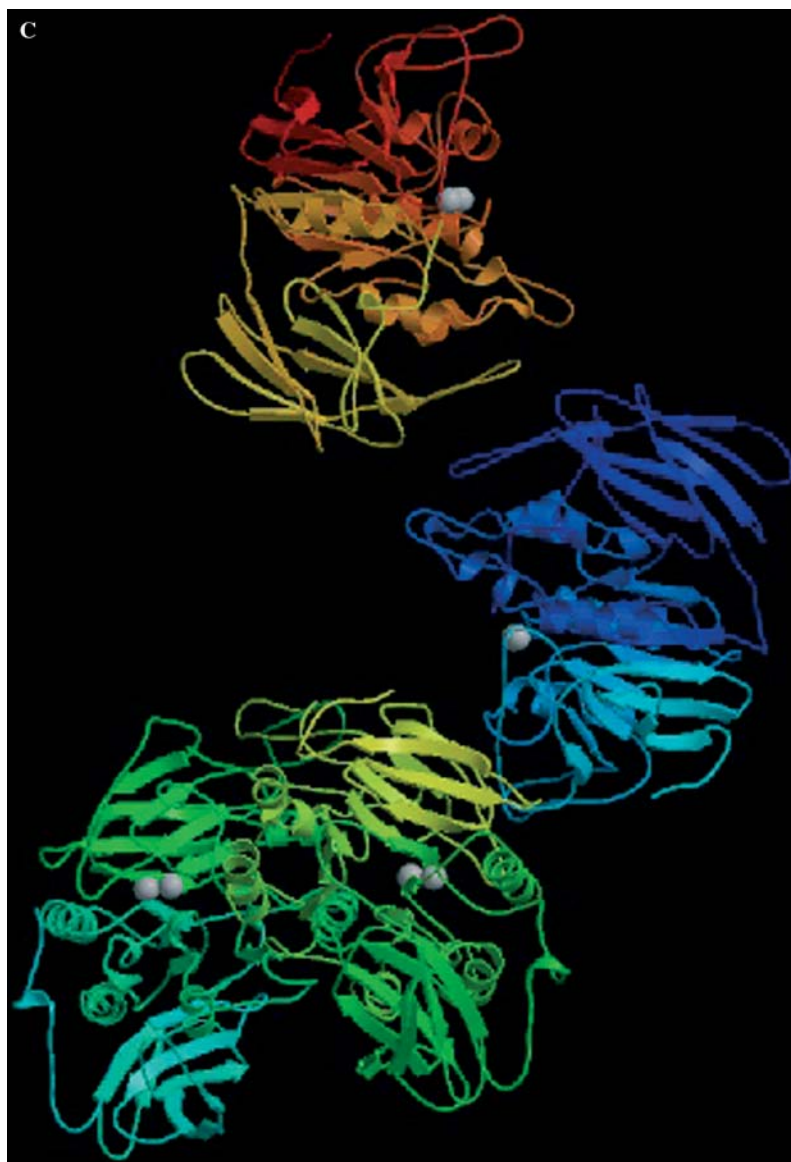


Fig. 1. (continued)

While BPP has no known homologous phosphatase, a recently developed method analyzing side-chain patterns of proteins by a “multidimensional index tree” has identified a class of enzymes, pyrophosphatase (PPase), that share some structural features with  $\beta$  propeller phytase [24]. An examination of these two classes of enzymes lends support to this sophisticated analysis. First, while phytases hydrolyze phytate, pyrophosphatases hydrolyze inorganic pyrophosphate. Second, the proposed catalytic mechanisms of these two enzymes are very similar. As in BBP, a water nucleophile that is coordinated to two metal ions in PPase attacks the phosphorus atom of its substrate. Moreover, both enzymes contain a “cleavage site” and an “affinity site.” The nucleophilic attack by the water molecule occurs in the former and the binding of a second phosphate group in the latter [24]. Whether BPP phytases are found in

other bacterial species or in eukaryotes remains to be determined.

#### **Purple acid phosphatase GmPhy—soybean (*Glycine max* L. Merr.) phytase**

Another phytase, GmPhy (EC 3.1.3.2), has recently been isolated from the cotyledons of germinating soybeans [25]. GmPhy has the active site motif of a purple acid phosphatase (PAP). This class of metalloenzyme has been widely studied [26]. Both its three-dimensional structure [27] and a proposed mechanism of catalysis are known [28]. Searches of genomic databases have revealed PAP-like sequences in plants, mammals, fungi, and bacteria [29]. The estimated size of purified GmPhy, 70–72 kDa, suggests a molecular mass similar to other



plant PAPs [25]. However, GmPhy is the only known PAP reported to have significant phytase activity. An *A. niger* NRRL 3135 PAP (Apase6) has previously been reported and displays only a minimum ability to utilize phytate as a substrate [30]. The lower catalytic activity of GmPhy than of *A. niger* NRRL 3135 phyA may be necessary during germination in soybeans because the germination process requires a steady breakdown of phytate over a period of several days [31]. While a three-dimensional model of GmPhy is not available, a model of another PAP, kidney bean purple acid phosphatase (KBPA), is available. Its NCBI structural number is 1KBP. Both GmPhy and kidney bean PAP share the purple acid phosphatase active site motif and a common catalytic mechanism. Amino acid sequence comparison revealed 33% homology between the two enzymes. Fig. 1C features (1KBP) to underscore the structural differences in these three classes of enzymes.

## Conclusion

The term “phytase” has become a broad rubric that includes several structurally different enzymes. A characteristic of one phytase will not automatically be applicable to all other enzymes collectively grouped under this name. Individual researchers have a responsibility to ensure that they clearly identify the specific enzyme and its class in all their communications. It is likely that additional classes of enzymes will be found to have phytate-degrading capabilities and also be labeled as a “phytase.”

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